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Impact of Age on Hypermutation of Immunoglobulin Variable Genes in Humans

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Chronological aging is associated with an accumulation of DNA mutations that results in cancer formation. The effect of aging on spontaneous mutations in humans is difficult to study because mutations are infrequent in the overall genome and tumors are relatively rare. In contrast, somatic mutations in immunoglobulin variable genes are abundant and can be studied in peripheral blood lymphocytes. To determine if aging alters the frequency and pattern of hypermutation, we sequenced 331 cDNA clones with rearranged V_H6 genes and compared 452 mutations from young humans to 570 mutations from old humans. There were more mutated clones in the young population compared to the old population. Among the mutated clones, the frequency, location, and types of substitutions were similar between the young and the old groups. However, the ratio of replacement-to-silent mutations was much higher in the complementarity-determining regions of heavy chains from old people, which indicates that their B cells had been selected by antigen. Among individuals, there was variability in the frequency of tandem mutations, which we have observed in mice defective for the PMS2 mismatch repair protein. Microsatellite variability in DNA, which is caused by impaired mismatch repair, was then measured, and there was a strong correlation between the frequency of tandem mutations and microsatellite alterations. The data suggest that individuals vary in their mismatch repair capacity, which can affect the mutational spectra in their antibodies.

KEY WORDS: Immunoglobulin genes; hypermutation; aging; antibody selection; mismatch repair.

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INTRODUCTION

In long-lived organisms, the genomic DNA of somatic cells gradually accumulates mutational changes with time. In humans, this mutational load contributes to the observed increase in cancer incidence with age, and probably to other age-related pathologies (1–3). Mutations throughout most of the genome arise as a consequence of DNA damage that is improperly repaired, as well as from the low intrinsic error rate of the DNA replication machinery. To limit the occurrence of such sequence changes, cells employ pathways such as base excision repair and nucleotide excision repair to remove damage caused by natural DNA decay and by reactive damaging agents of endogenous and extracellular origin (4). In addition, replication errors are minimized through an efficient postreplicative mismatch correction system (5). It is clearly desirable that all of these antimutagenic defense mechanisms are kept intact throughout the lifetime of an individual. If DNA repair were compromised with aging, this could contribute to an increased risk of cancer, as has been suggested from measurements of nucleotide excision repair and skin carcinogenesis in old humans (6).

A striking exception to the general need for limiting mutations is found in B lymphocytes, which use a highly specialized mechanism to introduce nucleotide substitutions into variable (V) genes of immunoglobulins (7). The hypermutation mechanism generates mutations up to a million times more frequently than those introduced by spontaneous mutation. This targeted, high-frequency form of mutagenesis has the purpose of increasing the repertoire of antibodies available for binding to antigens, which can challenge an organism anytime during its life span. Thus, it is preferable that this mechanism for generating antibody diversity is kept intact with age.

Several studies have examined V genes from old mice and humans to determine if they have hypermutation,

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and the results have been variable. Old mice do not mount a strong de novo immune response following deliberate immunization (8–10). However, B cells from Peyer's patches of old mice produce mutated antibodies (11), suggesting that memory B cells specific for environmental antigens are adequately stimulated in old animals (12). Different experimental approaches may explain the discrepancy in data from old humans. Thus, decreased frequencies of mutation were found in genes from B cells from nonvaccinated old subjects (13–15). In contrast, similar frequencies of mutation in young and old people were observed in cDNA clones of RNA made by activated cells (16–18) and from DNA from intestinal B cells responding to environmental antigens (19). Another confounding issue is that vaccination to influenza is common among the elderly population in the United States compared to Europe, and this may increase selection for specific antibodies. Furthermore, none of these studies made a detailed comparison of the spectrum of V gene mutations to see if the pattern and types of substitutions change with age.

The Baltimore Longitudinal Study on Aging provides a unique and valuable resource of documented blood samples from persons of different ages. Using this material, we collected a large database of V gene sequences from a sample of young and old humans, and performed an extensive analysis of the frequency and pattern of V gene mutations. We then searched for correlations between properties of the mutation pattern and microsatellite variability, which is an indicator of general genome instability.

MATERIALS AND METHODS

Study Participants

Five young and five old individuals, 26–29 and 81–86 years of age, respectively, were participants in the Baltimore Longitudinal Study on Aging program at the Gerontology Research Center, National Institute on Aging, National Institutes of Health, Baltimore, MD. The protocol was approved by the Institutional Review Board for Human Subjects Research of the Johns Hopkins Bayview Medical Center. Three people in the young group, Y1, Y3, and Y4, and four people in the old group, O1, O3, O4, and O5, were females. None of the participants expressed an acute illness at the time of blood removal. Subject Y5 was under treatment for asthma and allergic skin reactions with histamine and steroid creams, and subject O5 was under treatment for polymyalgia rheumatica with oral prednisone. The five old people were immunized against influenza during the 10 months prior to blood removal; the young people were not immunized during that time.

Blood Collection, RNA and DNA Preparation

Twenty milliliters of peripheral blood was collected in EDTA. Mononuclear cells were isolated by centrifugation through Ficoll–Paque Plus (Amersham Life Science Inc., Arlington Heights, IL), and total RNA was extracted using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). To make cDNA, 0.25 μg of RNA was transcribed with Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and a reverse primer complementary to the mRNA starting at codon 264 in the C_H2 exon of the constant (C) gene for IgM (20), 5′AAGAAGCCGTCGCGGGGTGG. DNA was extracted from a portion of the cells with phenol and chloroform, precipitated with ethanol, and resuspended in 50 μg per ml of 10 m*M* Tris–1 m*M* EDTA, pH7.6.

cDNA Cloning and Sequencing

The cDNA was amplified in a 50-μl reaction containing half of the cDNA, *Pfu* DNA polymerase (Stratagene, La Jolla, CA), a forward first primer for the leader region of the V_H6 gene starting at codon −19 (21), 5′TCTGTCTCCTTCCTCATCTTC, and the reverse first primer shown above. The amplification consisted of 30 cycles of denaturation at 95°C for 1 min, annealing at 64°C for 2 min, and extension at 72°C for 3 min, followed by a final incubation at 72°C for 10 min. Two microliters of the reaction was then amplified for another 30 cycles using a second set of nested primers containing restriction sites for cloning. The forward second primer started at codon -10 in the leader region and contained a *Bam*HI site, 5′CGCGGATCCGCCCGTGCTGGGCCTCCCATG; and the reverse second primer started at codon 223 in C_H1 in the C_μ gene and contained a *Hin*dIII site, 5′TGGAAGCTTCACGTTCTTTTCTTTGTTGCC. The 670-bp PCR products containing both V and C genes were cloned into restriction-digested M13mp18, and viral plaques containing inserts were identified by hybridization to a V_H6-specific oligonucleotide. Viral DNA with rearranged V_H6 genes were sequenced with a primer starting at codon 140 in C_H1 of the Cμ gene, 5′AACGGCCACGCTGCTCGTATC, and with successive primers located across the V_H6 gene. C_H1 exons were sequenced with a M13 primer starting at the *Hin*dIII site.

Microsatellite Variability

The concentration of DNA from peripheral blood lymphocytes was determined by optical density, and samples were diluted to 5 pg/μl. The D2S123 microsatellite containing a dinucleotide repeat on chromosome 2 was amplified with a forward primer, 5′AAACAGGATGCCTGCCTTTA, and a reverse primer, 5′GGACTTTCCACCTATGGGAC (22). To detect accurately an infrequent change in size of a microsatellite, the DNA concentrations were adjusted so that only one positive PCR product was obtained in three reactions. The 20-μl reactions contained 1–10 pg of DNA, 80 ng of the forward primer, 80 ng of the reverse primer, 1 nmol each of dATP, dCTP, dGTP, and dTTP, 0.66 pmol of [$\alpha-^{32}$P]dCTP at 3000 Ci/mmol (Amersham Life Science Inc., Arlington Heights, IL), 0.2 μl of Platinum *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD), and 1× PCR buffer with 30 nmol of $MgCl_2$. The DNA was amplified in 96-well plates for 30 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C for 35 cycles. The products were separated on 6% denaturing acrylamide gels with 20% formamide and visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Only gels with 35% or less positive lanes were analyzed. The positive reactions were then loaded next to each other on a second gel and separated to identify band shifts.

Statistical Analyses

The analysis of tandem substitutions is based on comparing the observed number of adjacent mutations to the statistical probability that two mutations will randomly occur next to each other; thus, a large number of mutations in a segment increases the probability for adjacent mutations to occur by chance, and a low number of mutations decreases that probability. The formula for the expected number of tandem substitutions when n mutations are randomly distributed in a sequence of k consecutive nucleotides is equal to $n(n-1)/k$ (23). k for each FR segment was 90 (FR1), 42 (FR2), 96 (FR3), and 33 (FR4). The comparison of the observed number to the expected number for each age group was based on exact Poisson calculations. Comparisons of the frequency distributions by type of substitution between young and old individuals were performed using Pearson's chi-square test. Because of variability among individuals within age groups, comparisons of mutation frequencies, microsatellite alterations, and the ratios of observed to expected numbers of tandem mutations between old and young individuals were made using the t test. Pearson correlation coefficients and Spearman rank correlation coefficients were calculated to measure the relationship between different parameters, and exact tests were employed to determine if correlation coefficients were significantly different from zero. Two-sided P values are reported for all comparisons.

RESULTS

The rationale of this study was to compare a large database of mutations from two widely separated age groups to see if age had any effect on somatic hypermutation. Peripheral blood was collected from five people between 26 and 29 years old and five people between 81 and 86 years old, and approximately 25 clones per young individual and 40 clones per old individual were sequenced from cDNA libraries. We focused on the rearranged V_H6 gene associated with IgM heavy (H) chains for the following reasons. First, the V_H6 gene segment is nonpolymorphic (24) and mutations can be easily identified. Second, IgM H chains, which are encoded by the Cμ constant gene, are expressed by newly- generated and long-lived B cells in humans, and the analysis would include both de novo and memory mutations.

Frequency of Mutations

One hundred twenty-eight clones from five young and 203 clones from five old people were analyzed for mutations in the V and J gene segments encoding framework regions (FR) 1–4 and complementarity determining regions (CDR) 1 and 2. Mutations in CDR3 were not identified because of unknown variability introduced during joining of the gene segments. The nucleotide sequences were compared to the germline sequences of the V_H6 gene segment (21), J_H gene segments (25), and the C_H1 exon of the Cμ gene (20) to identify somatic mutations. Mutations at the 3′ and 5′ ends of V and J gene segments were assigned if they were preceded by two or more consecutive germline nucleotides.

Clones were classified as follows: (a) hypermutated clones had two or more mutations and unique CDR3s, (b) related clones had the same CDR3 sequence with both shared and unique mutations, (c) nonhypermutated clones had zero or one mutation and unique CDR3s, (d) duplicated clones were identical, and (e) hybrid clones from PCR amplification had portions of sequences from different genes. Around 2% of the cDNA clones were nonproductive with stop codons and frameshifts in CDR3 and were not included in further analyses. Of the 126 productive clones sequenced from five young humans, 43% were hypermutated, 13% were related, 27%

were nonhypermutated, 11% were duplicated, and 6% were hybrids. Of the 197 productive clones sequenced from five old humans, 29% were hypermutated, 4% were related, 49% were nonhypermutated, 15% were duplicated, and 3% were hybrids. Among the young people, the percentages of sequenced clones with hypermutation were 28.1, 34.5, 57.9, 56.5, and 47.8% for Y1 through Y5, respectively, with a mean percentage of 45.0% (±5.9%). Among the old people, the percentages were 11.9, 27.8, 22.2, 38.5, and 60.0% for O1 through O5, respectively. If individual O5, who was taking prednisone, is excluded, the mean percentage of mutated clones among the old people is 25.1% (±1.2%), which is significantly lower than that for the young group ($P = 0.048$).

Only the hypermutated clones were considered for further analysis and are listed in Table I. Some 54 clones from young humans and 58 clones from old humans were studied; the number of mutations per clone ranged from 2 to 30. The overall frequency of mutation per base pair for clones from young individuals was 2.4%, and that for clones from old individuals was 2.8%, which is within the normal range of mutations in IgM molecules from peripheral blood (26, 27). There was no statistical difference between these two groups ($P = 0.20$). However, the frequencies were quite homogeneous within the young group ($P = 0.23$) but heterogeneous within the old group ($P < 10^{-6}$). Subject O5 had the lowest frequency, which may be a result of taking prednisone. If O5 is excluded from the analysis, the frequencies are higher for the old population ($P = 0.05$), which could be due to some aspect of aging or influenza vaccination. As a control for *Pfu* polymerase error, the C_H1 exon of the $C\mu$ gene was sequenced from 33 clones that had mutations in their V genes. No mutations were found in 6543 bp, corresponding to $<2.5 \times 10^{-6}$ mutations per bp per PCR cycle, which is about 200-fold lower than the frequency of mutations in the V genes. Some 99.7% of the mutations were nucleotide substitutions; the rest were one insertion of three nucleotides and two deletions of three and six nucleotides in CDR1, which kept the translation in reading frame. Infrequent insertions and deletions in CDRs 1 and 2 of human V genes have been reported before (28–30) and may be the result of slippage of DNA polymerase during replication or repair.

Table I. Number of Mutations in Rearranged V_H6 Genes from Young and Old Humans

Subject	Clone	Number[a]	Subject	Clone	Number
Y1	5122	2	O1	50	2
	5062	4		5	5
	5102	5		82	12
	5123	5		33	21
	5070	6		66	24
	5109	7	O2	37	3
	5035	10		1	7
	5023	11		111	8
	5067	26		21	8
Y2	J016	2		106	8
	J017	3		13	8
	J008	4		191	9
	J031	6		180	12
	J009	6		10	12
	J027	10		34	15
	J037	12		4	21
	J001	14		9	21
	J036	14		88	22
	J013	18		25	22
Y3	4158	2		167	30
	4073	2	O3	6154	3
	4112	2		6074	7
	4086	3		6066	7
	4111	6		6010	7
	4007	6		6052	9
	4001	7		6076	10
	4071	7		6081	12
	4072	8		6018	13
	4136	12		6077	18
	4123	24		6079	18
Y4	7182	3	O4	8004	2
	7011	3		8039	4
	7168	4		8044	4
	7183	4		8003	5
	7160	5		8033	5
	7004	7		8024	7
	7149	7		8007	9
	7177	8		8010	11
	7124	8		8050	13
	7113	11		8043	20
	7107	14	O5	9011	2
	7150	14		9039	2
	7155	19		9028	2
Y5	10	3		9066	2
	2	3		9008	3
	N90	3		9052	4
	N78	3		9072	4
	N15	7		9053	5
	N86	7		9041	7
	N75	10		9067	7
	L79	10		9009	8
	N81	16		9042	9
	L53	18		9029	9
	N66	21		9020	10
				9022	10
				9099	10
				9074	11
				9101	11

[a]Mutations were recorded in rearranged V_H6 and J gene segments; the average length was 347 bp per clone. The average frequency of mutations per base pair for young subjects Y1–Y5 was 2.4% (452 mutations in 18,738 bp), and that for old subjects O1–O5 was 2.8% (570 mutations in 20,126 bp).

Distribution of Mutations in the V and J Gene Segments

The position and types of mutations in the V_H6 gene segment are shown in Fig. 1, which demonstrates the

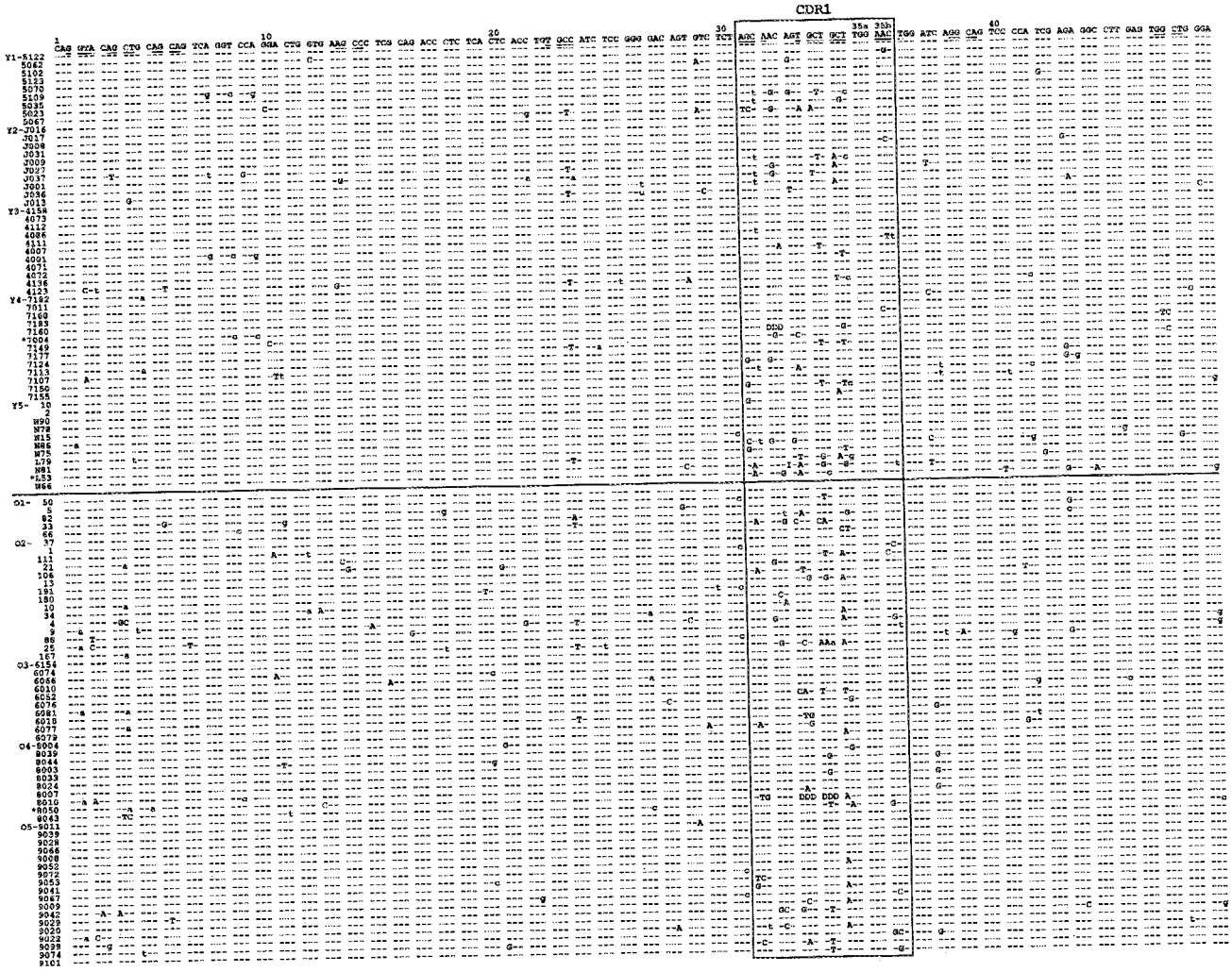

Fig. 1. Nucleotide sequences of rearranged V_H6 gene segments in clones from young and old humans. Clones from young individuals are listed above the horizontal line in the top half of the figure, and clones from old individuals are shown below the line. Codon positions are numbered and CDRs are depicted according to Ref. 20. The hot-spot motifs, RGYW and its inverse complement, WRCY, are underlined in the germline sequence. A dash indicates identity to the germline sequence, replacement substitutions are in uppercase, and silent substitutions are in lowercase. (*) Clones with deletions (D) and insertions (I).

tremendous diversity that is generated by the hypermutation process. Substitutions are noted as either replacement changes, if they change the codon to specify a different amino acid, or silent changes, if they do not change the codon. An illustration of the frequency of mutations per codon is shown in Fig. 2. The distribution of mutations in clones from young people is mirrored by the distribution in clones from old people and shows no difference in the number of mutations and their location in the V gene segment.

Mutations in the J gene segments are shown in Fig. 3. In clones from both young and old individuals, J_H4 was predominantly utilized, and J_H1 and J_H2 were rarely used, which is consistent with previous studies (18, 25, 31). The overall frequency of mutation per base pair in the FR and CDR regions for both groups is as follows: FR1, 1.2% (123 mutations in 10,170 bp); CDR1, 6.3% (149 mutations in 2373 bp); FR2, 1.1% (51 mutations in 4746 bp); CDR2, 4.8% (293 mutations in 6102 bp); FR3, 2.8% (299 mutations in 10,752 bp); the portion of the J gene segments that comprise CDR3, 4.7% (68 mutations in 1458 bp); and FR4, 0.9% (34 mutations in 3729 bp). Within these regions, there are subregions of hypermutability occurring at residues 31–35 in CDR1 (7.9% mutations/bp), residues 56–61 in CDR2 (9.6% mutations/bp), residues 67–71 in FR3 (6.2% mutations/bp),

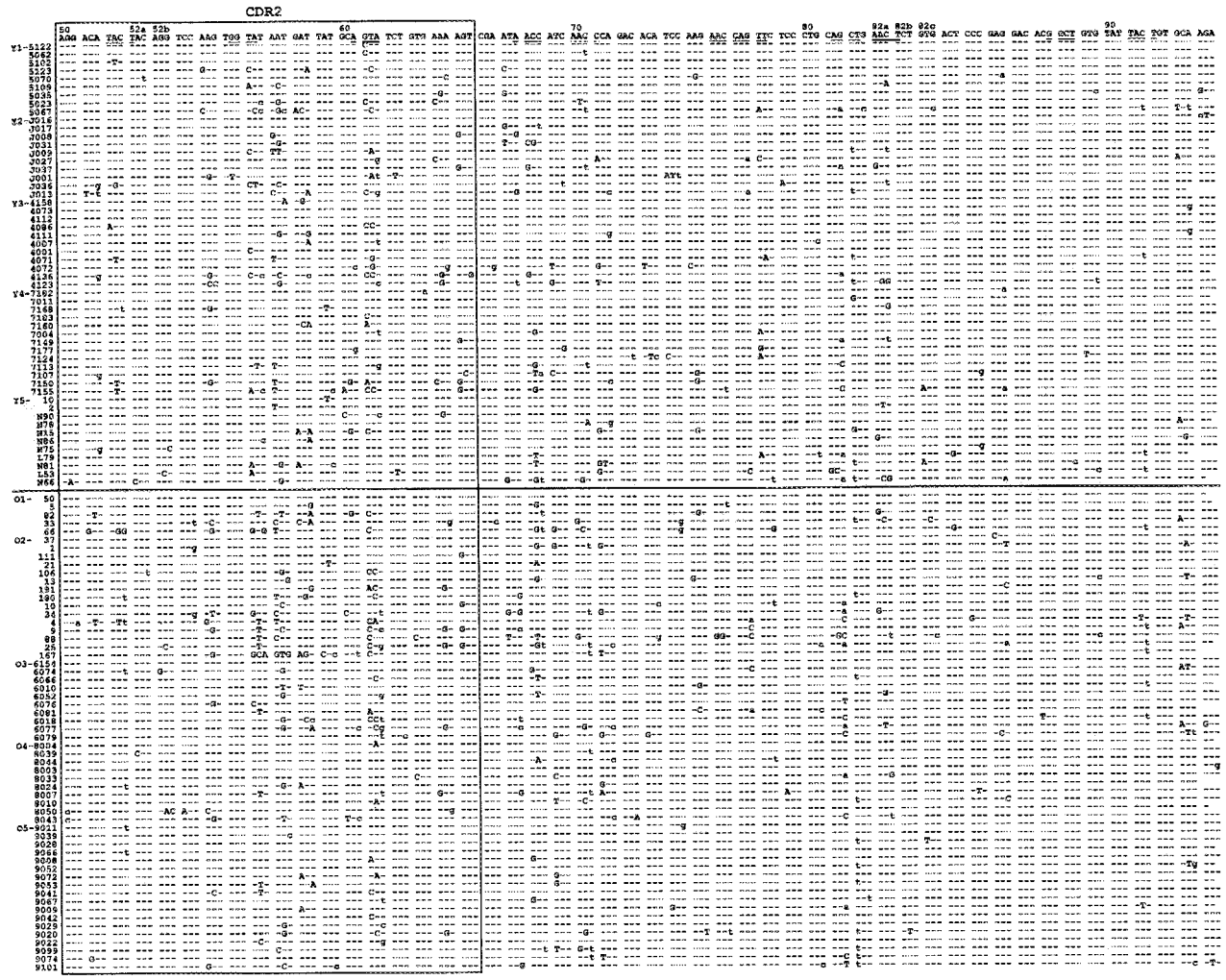

Fig. 1. (Continued).

and residues 81–82a in FR3 (6.6% mutations/bp). FR residues 67–71 may have more mutations because they are proximal to the antigen binding site (32), and residues 81–82a may be hypermutable because they contain nucleotide sequences (33–35) that are associated with increased mutations: RGYW (R=purine, A or G; Y=pyrimidine, C or T; W = A or T) and its inverse complement, WRCY. Recently, DNA double-strand breaks have been frequently detected at these hot spots (36, 37), which suggests that they may be recognized by an endonuclease during hypermutation.

Types of Substitutions

The types of substitutions as recorded from the coding strand are listed in Table II. There were 306 replacement and 144 silent mutations for the young group, and 397 replacement and 172 silent mutations for the old group, with no difference between young and old in the types of substitutions ($P = 0.59$). For a mutational analysis that is not biased by selection, we studied silent substitutions in the eight codons that can use all four nucleotides in the third position; i.e., any of three nucleotides can be substituted without changing the amino acid. In this category, there were approximately 55% transitions and 45% transversions in both groups of clones ($P = 0.9$), and there was no difference in the types of substitutions ($P = 0.63$). A was mutated about 36% of the time, T was mutated 30%, C was mutated 14%, and G was mutated 20%. The preference for silent mutations of A and T compared to G and C may be particular to the V_H6 sequence (38) since a similar analysis of mutations in V_H4 genes (39) did not show this pattern.

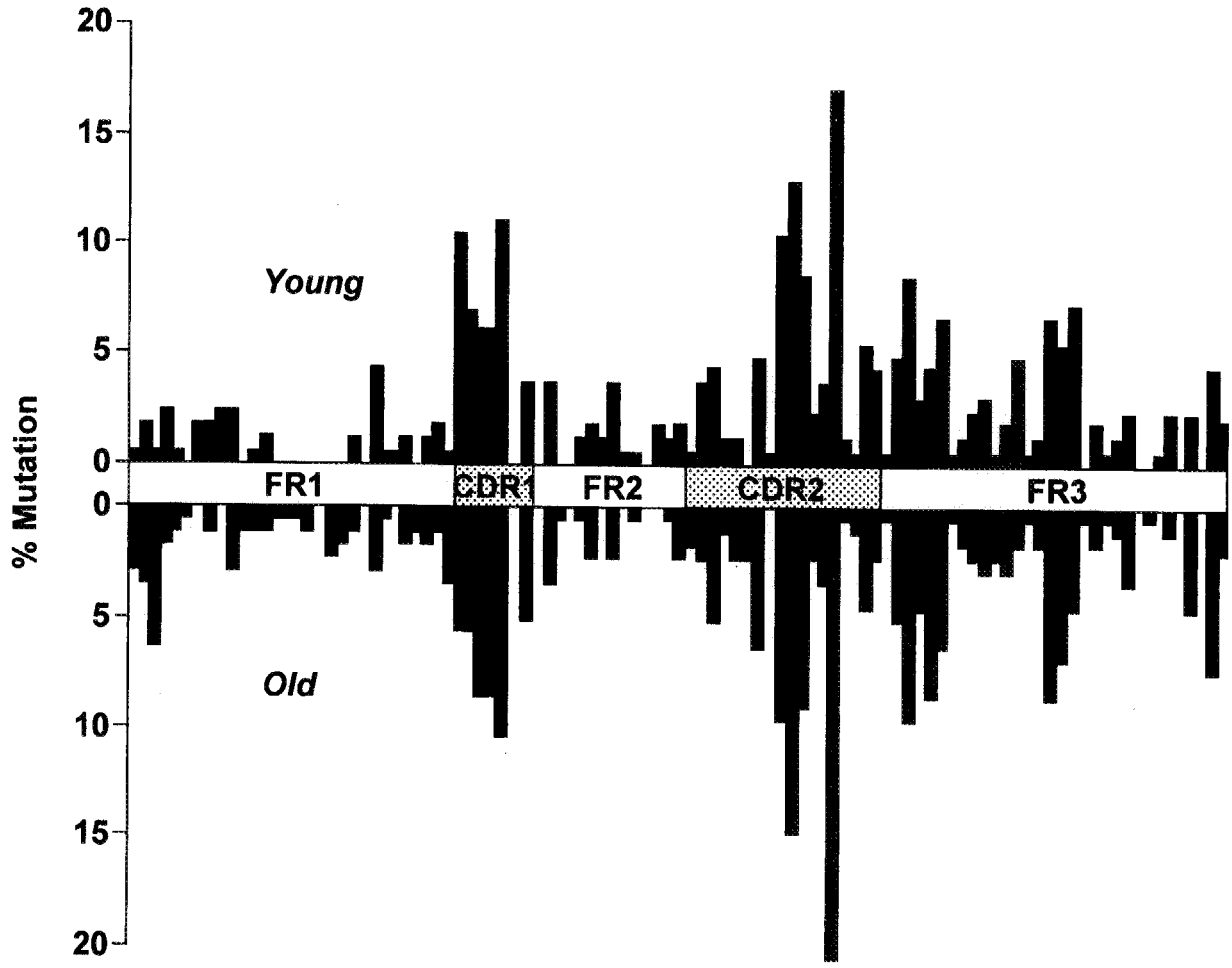

Fig. 2. Average mutational frequency per codon in V_H6 gene segments from young and old humans. Bars represent the percentage mutation calculated by the number of mutations per codon, divided by 3 (nucleotides), divided by the number of clones, multiplied by 100.

Ratios of Replacement-to-Silent Mutations

The ratios of replacement mutations to silent mutations are higher in the CDRs than in the FRs as a result of selection for B cells exhibiting immunoglobulin receptors with higher affinity for antigen. In Fig. 4, the replacement-to-silent ratios are shown throughout the V gene segment for clones from the young and old groups. V_H6 genes from the old population showed a dramatic increase in replacement changes in CDRs 1 and 2 compared to those from the young population. The differences were significant at residues 31–35 in CDR1 ($P = 0.004$) and residue 56 in CDR2 ($P = 0.045$). Furthermore, a specific tyrosine-to-phenylalanine change at residue 56 occurred nine times in clones from all five old humans and only once in a clone from a young human ($P = 0.015$).

Tandem Mutations and Microsatellite Variability

We have observed an increased frequency of adjacent mutations in V genes from mice deficient for the PMS2 mismatch repair protein (23). To see if tandem mutations were significantly found in V genes from humans, the observed numbers of tandem mutations were compared to the expected numbers (see Materials and Methods). Only mutations in FRs 1–4 were analyzed, since they are not positively selected for binding to antigen. As shown in Table III, the ratios of observed to expected tandem pairs varied among the individuals but did not differ between the young and the old groups ($p=0.31$). Triplet substitutions were also observed in the sequences but were too infrequent to perform a statistical analysis.

If the accumulation of tandem mutations is related to defective mismatch repair, then the individuals with the

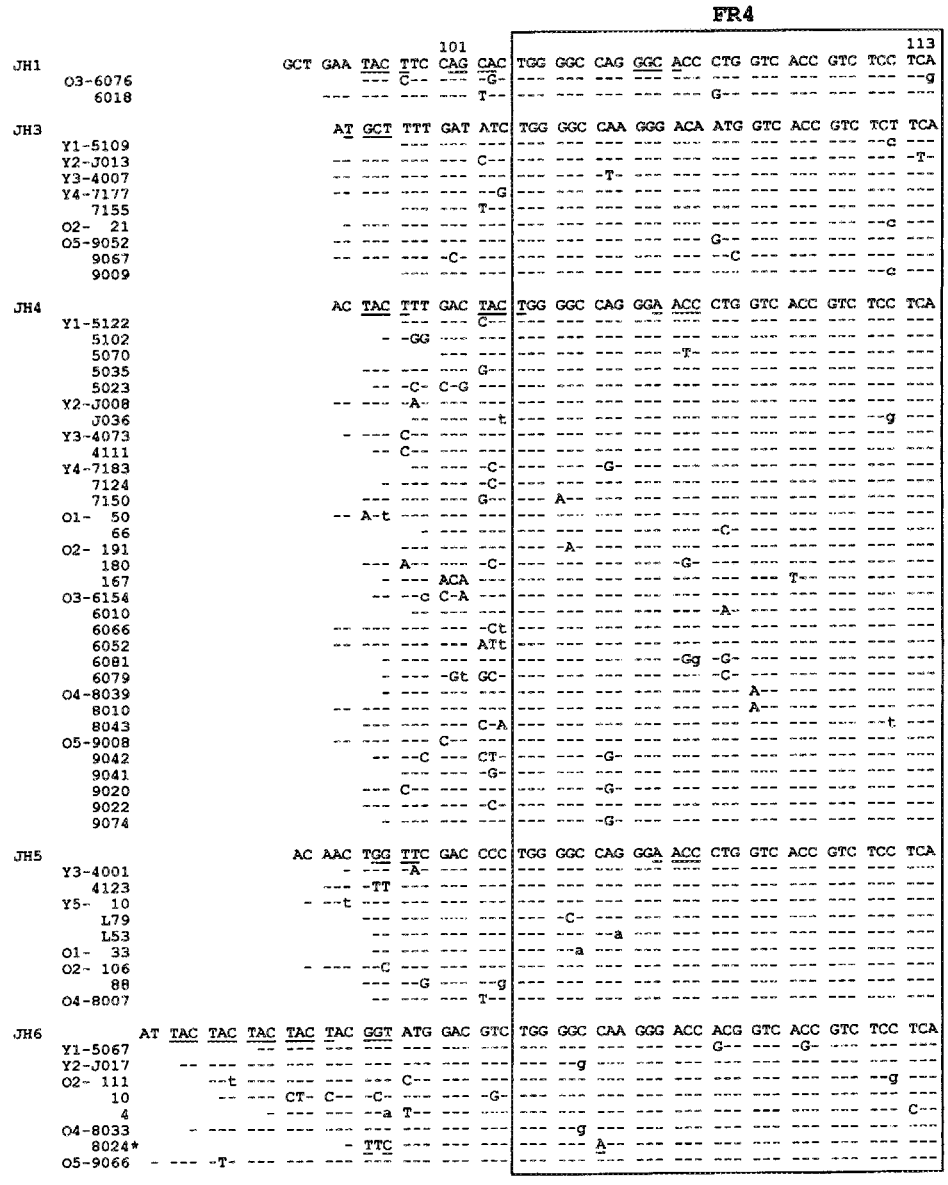

Fig. 3. Mutations in J_H gene segments. Conventions are as in Fig. 1. FR4 is boxed, and (*) A clone with a different allele of J_H6, with the allelic nucleotides underlined (25).

highest frequency of tandem mutations should also have increased microsatellite variability. Alterations were analyzed in the CA dinucleotide repeat of the D123S microsatellite on chromosome 2 using DNA from peripheral blood lymphocytes of both the young and the old groups. Examples of altered microsatellites are shown in Fig. 5, which also illustrates the polymorphism at this allele. Individuals Y1, Y3, Y4, Y5, and O3 were homozygous for the locus; Y2, O1, and O2 had alleles that differed by 2 bp; O5 had alleles that differed by 4 bp; and O4 had alleles that differed by 10 bp. Insertions and deletions of 2 bp (74% of the alterations), 4 bp (15%), and 6 bp (11%) were observed, with deletions occurring twice as frequently as insertions.

The data in Table III show a range of microsatellite variability of 1–6% in the 10 individuals. The assays were repeated several times, with similar results. Microsatellite changes in the young group did not differ from the old group ($P = 0.31$). The tandem ratios were then plotted against the frequencies of altered microsatellites in all 10 humans, shown in Fig. 6. The relationship was linear and highly significant, with a Pearson correlation

Table II. Types of Substitutions in Rearranged V_H6 Genes[a]

Substitution	% total[b] Young (n = 450)	% total[b] Old (n = 569)	% silent[c] Young (n = 68)	% silent[c] Old (n = 67)
A to				
G	20	20	24	13
T	9	8	8	5
C	9	10	9	13
T to				
C	10	8	24	26
A	6	4	0	5
G	4	3	4	0
C to				
T	14	14	4	8
A	1	3	3	1
G	7	7	4	8
G to				
A	9	11	4	8
T	3	3	4	3
C	8	9	12	10

[a]Data are corrected for base composition, so that the numbers represent the percentage substitutions expected from a sequence with equal numbers of A, T, C, and G.
[b]Includes all replacement and silent substitutions.
[c]Silent substitutions in the eight codons with threefold wobble in the third position.

coefficient of 0.91 (P = 0.0004); the Spearman rank correlation gave similar results. Table III also summarizes the mutation data per individual; there was no significant correlation between the frequency of microsatellite changes and either the mutation frequency (Pearson correlation coefficient of 0.25, P = 0.48) or the percentage of G and C mutations (Pearson correlation coefficient of 0.06, P = 0.85).

DISCUSSION

Old Humans Express Highly Mutated Antibodies

Young humans had more hypermutated cDNA clones than old humans, confirming that some aspects of cellular immunity decline with age (reviewed in Ref. 40). However, among the mutated clones, the frequencies of mutation were very similar between the young (2.4% mutations/bp) and the old (2.8% mutations/bp) groups. The frequency of mutation in rearranged V_H6 gene segments is similar to that obtained in a study of other genes in V_H families 1–7 (18) and, therefore, reflects a general property of V_H genes in IgM transcripts from peripheral blood B cells. The location of mutations in the FRs and CDRs and the types of substitutions were also identical between the two groups. Thus, even though the old population had fewer B cells with mutation, it is likely that they have the ability to mount an adequate humoral immune response because of strong selection for those cells expressing mutated antibodies.

It is not known whether the mutations in clones from old people were induced by the mutation mechanism in naive B cells or were already present in long-lived memory cells. There is evidence supporting both pathways in humans. B cells with unmutated V genes are frequently found in the peripheral blood of old people (13, 18), and they likely arise from newly generated cells in bone marrow (41). Long-lived memory B cells expressing IgM with heavily mutated genes are also readily detected in peripheral blood (42, 43). Another possibility is that V genes in memory cells undergo further mutation upon repeated rounds of antigen encounters (11). The data in this report are consistent with participation from both naive and memory cells in old people. Thus, the clones in Fig. 1 with a few mutations and no replacement changes in CDRs1 and 2 may have derived from recently mutated, unselected B cells. The clones with many mutations may have come from memory cells that have undergone years of selection and expansion for binding to antigen.

Antibodies from Old Humans Are Highly Selected in CDRs

Although the young and old groups had the same frequency of mutation in CDRs and FRs (Fig. 2), selection for those substitutions leading to replacement amino acids in the CDRs was strikingly enhanced in genes from the old group (Fig. 4). The cDNA clones likely came from the abundant RNA made by plasma cells that were generated from recently- stimulated naive or memory B cells (44). It is not known if this difference is due to aging or recent exposure to antigen. The prevalence of nine tyrosine-to-phenylalanine changes at residue 56 in CDR2 in all five old individuals compared to only once in a young person suggests that the old group was exposed to at least one common antigen that the young group did not encounter. The specificity of these V_H6-encoded antibodies is unknown since the corresponding light chains were not identified. In general, V_H6-encoded antibodies are polyreactive and have been shown to bind bacteria, DNA, and cardiolipin (45–47), suggesting that the old people were undergoing an autoimmune reaction. It is also possible that some of these antibodies bind influenza antigens, since the old people recently received influenza vaccinations and the young people did not. The vaccination status would also explain why a similar study on V_H6 genes in unimmunized old subjects failed to show selection for replacement changes in the CDRs (12). However, the age-

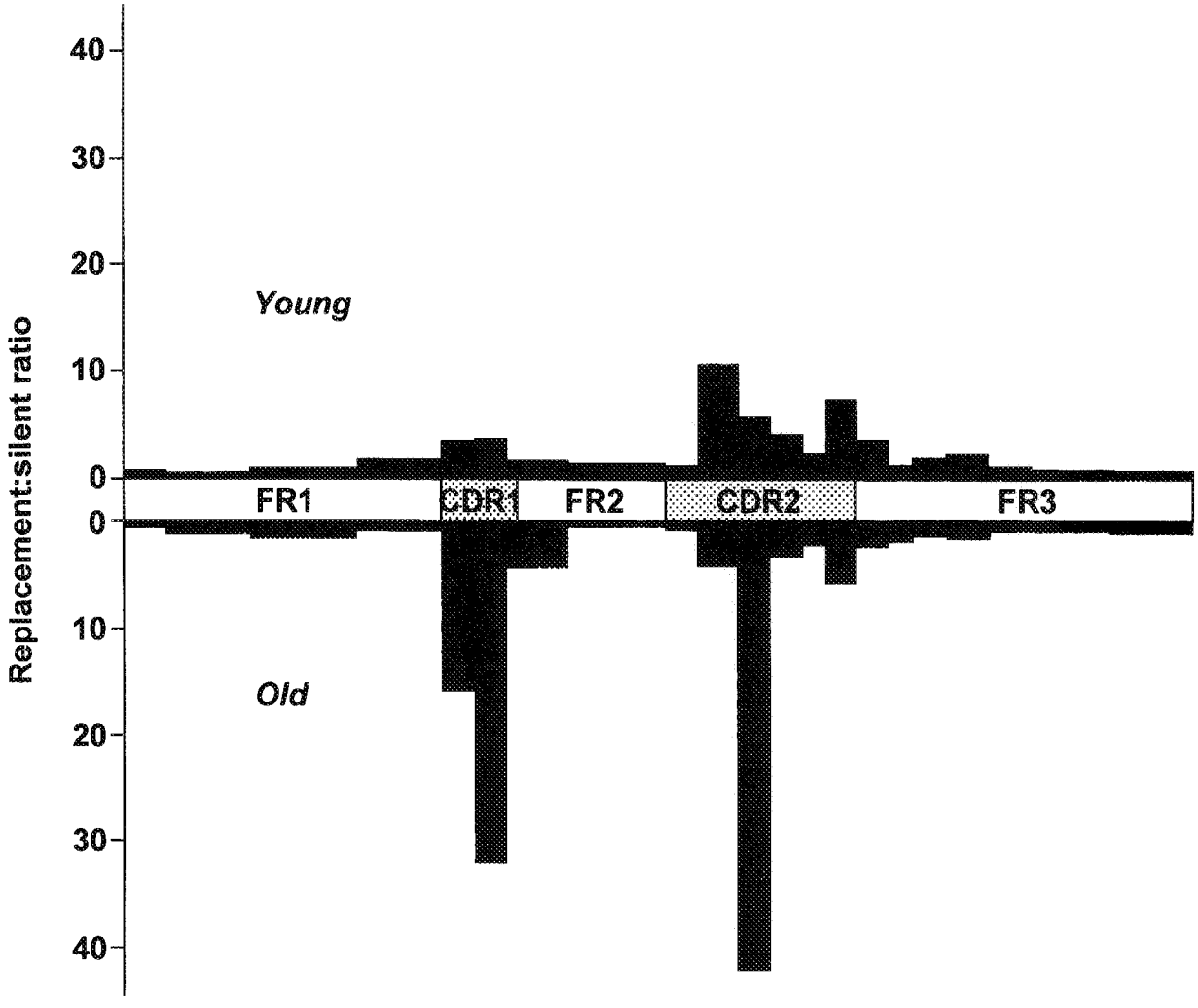

Fig. 4. Replacement-to-silent substitution ratios in V_H6 gene segments in clones from young and old humans. Bars represent the average ratios calculated for 2–10 codon increments.

related impact of immunization on the mutation frequency cannot be evaluated in the present study because the young subjects were not vaccinated.

Tandem Mutations in V Genes Correlate with Microsatellite Variability

Recent experiments by several groups have reported altered patterns of mutation in V genes from mice deficient for several mismatch repair proteins. Mice deficient in MSH2, a protein that binds to mismatches, have an increased number of mutations of G and C nucleotides (48–51), and we observed that mice deficient in PMS2, a protein that combines with MSH2 and other proteins to excise mismatches, have an increased number of tandem mutations (23). Other groups studying mutation in PMS2-deficient mice did not detect an increased number of tandem mutations (50, 52–54); however, their data were limited to 3–13 clones per study with two or more mutations, whereas our analysis included 30 clones. The data suggest that an error-prone DNA polymerase functions during hypermutation to generate mismatched base pairs, and some of these are corrected by the mismatch repair pathway. To see if the mutational spectra changes with age in humans, we analyzed unselected silent and FR mutations for these two parameters. There was no difference between the young and the old groups in the frequency of mutations of G and C or in the frequency of tandem pairs. However, since there was a broad range in the data, a correlation with mismatch repair may exist on an individual basis.

Lymphocyte DNA was then examined for microsatellite variability, which is known to accumulate in cells with defective mismatch repair (5). Insertions and dele-

Table III. Hypermutation and Microsatellite Changes per Individual

	Total frequency mutations/bp (%)	Silent mutations of G and C[a] (%)	FR tandems (observed/expected) ratio	Microsatellite variability, % (altered/total)[b]
Young				
Y1	2.4	36	0 (0/0.86)	1.3 (1/79)
Y3	2.1	13	0.8 (1/1.28)	2.2 (2/92)
Y4	2.4	43	1.6 (2/1.22)	3.9 (3/77)
Y5	2.6	25	2.0 (5/2.48)	3.4 (3/87)
Y2	2.6	56	3.1 (3/0.96)	3.4 (3/89)
Old				
O1	3.7	51	0 (0/1.22)	1.1 (1/93)
O3	3.0	26	1.9 (3/1.62)	2.9 (2/68)
O2	4.0	49	2.0 (9:4.54)	4.4 (4/91)
O4	2.3	38	4.4 (4:0.92)	5.6 (4/72)
O5	1.9	32	4.9 (6/1.22)	5.3 (4/76)

[a]From Table II.
[b]Number of reactions with microsatellite bands of different mobilities divided by total number of positive reactions.

tions are occasionally introduced during replication when DNA polymerase slips on repetitive sequences and misaligns on the template, and the length variations are usually removed by the mismatch repair pathway. If the pathway is compromised, the insertions and deletions remain and are copied into the cell's genome. An analysis of the length variation of the S123 dinucleotide satellite marker revealed a range of 1–6% alterations in peripheral blood cells from both young and old populations, with no difference between the two groups. This detection of microsatellite variability in a heterogeneous population of B and T cells from healthy donors is remarkable considering that microsatellite variations in T-cell clones from healthy individuals is only 0.3% (55). Per individual, there was no correlation between the frequency of altered microsatellites and mutations of G and C. However, a plot of the frequency of altered microsatellites vs tandem mutations in all 10 subjects showed a very significant correlation ($P < 10^{-3}$; Fig. 6), which indicates that the two phenomena are related. Thus, an increased frequency of tandem mutations may serve as a marker for altered mismatch repair function.

Tandem Mutations May Be Generated During One Passage of an Error-Prone DNA Polymerase

Base substitutions have been shown to accumulate at the nucleotide motifs RGYW and its inverse complement, WRCY (33–35). Approximately 24% of the nucleotides in FRs 1–4 in V_H6 and J_H gene segments are in

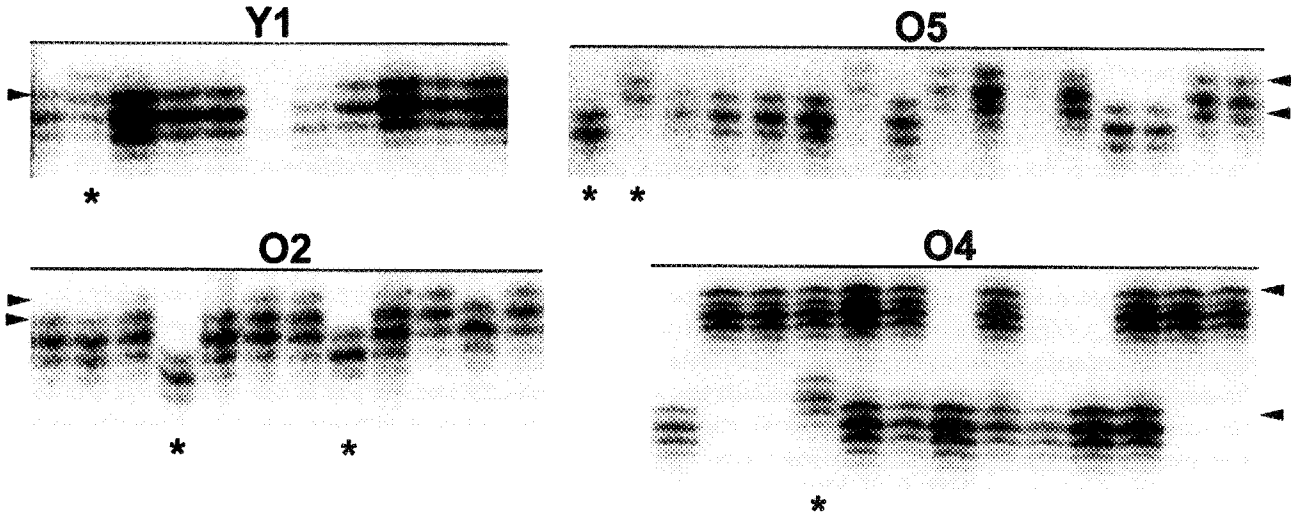

Fig. 5. Microsatellite variability in peripheral blood DNA. Examples of variability and polymorphism for the S123 locus are shown for subjects with homozygous alleles (Y1) and heterozygous alleles differing by 2 bp (O2), 4 bp (O5), and 10 bp (O4). Arrowheads indicate the position of the major PCR products from undiluted template DNA, and asterisks mark the lanes with insertions or deletions.

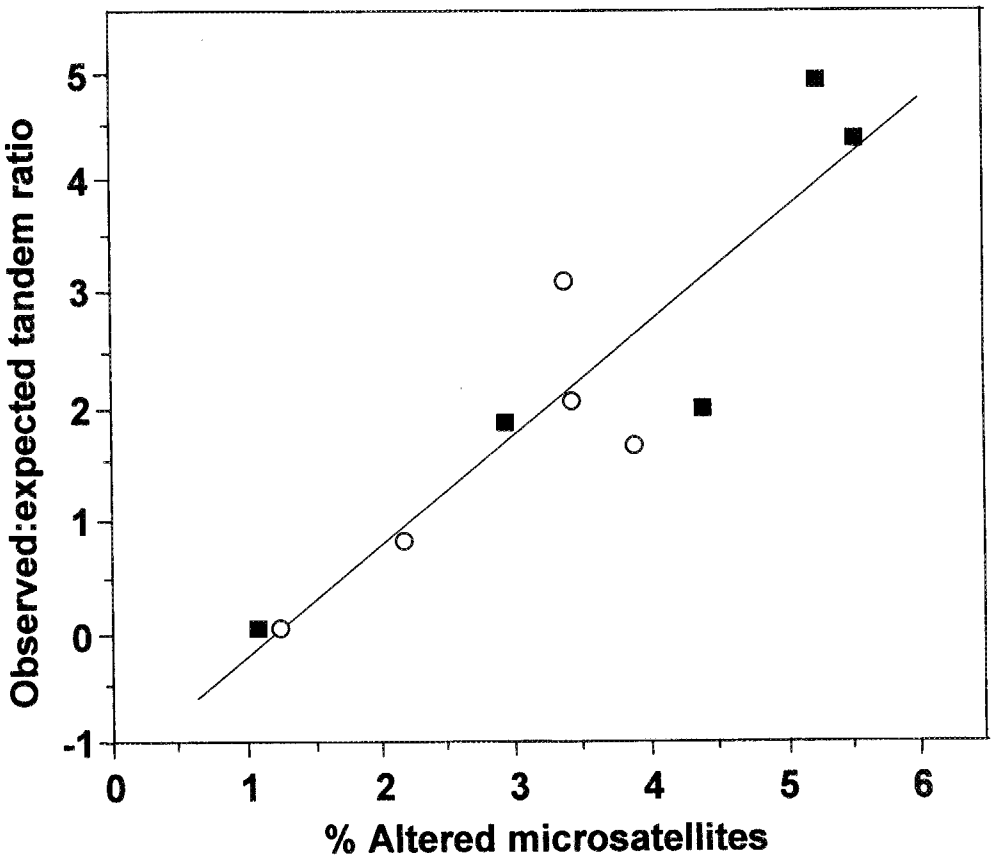

Fig. 6. Correlation of tandem mutations and microsatellite variability. Values from young individuals are shown by open circles, and values from old individuals are shown by filled squares.

RGYW/WRCY sequences (underlined in Figs. 1 and 3), whereas 70% (23/33) of the tandem pairs were in these sequences. This indicates that the tandem mutations were targeted to the hot spot motifs. Adjacent mutations may occur consecutively during each round of repair and/or simultaneously during one round of repair (56). The following reasons imply that many of them could be generated during one passage of an error-prone DNA polymerase. First, if two mutations occurred singly within the four-4 nucleotide motif, half of the mutations would be tandem by chance. Within this data set, 12.5 tandem mutations would be expected to occur by chance, whereas 23 were observed. Thus, tandem mutations occurred significantly more often than would be expected from two individual events ($P < 10^{-4}$), suggesting that many of them arose from a single event. Second, a single mutation may destroy an existing hot spot or create a new one. For example, clone O4-8043 has a tandem mutation, TC, at codon 3 within an RGYW hot spot (Fig. 1). Both mutations would destroy the hot spot if they occurred singly: if the T mutation occurred first, the sequence would become TGYW, and if the C mutation occurred first, the sequence would become RCYW. Alternatively, clone Y4-7107 has a tandem mutation, TT, at codon 10 that is not in a hot spot. The first T mutation would create a RGYW hot spot that could attract the second T mutation. The data in this study were analyzed by these parameters. If paired mutations occurred one at a time within a hot spot, 33% of the mutations would keep the motif and 67% would destroy it. If they occurred singly outside of a hot spot, 20% would create a new hot spot and 80% would not. Thus, two successive mutations are more likely to destroy a hot spot than to keep the motif or create a new hot spot. Third, some of the newly described DNA polymerases are able to catalyze two adjacent misincorporations into undamaged DNA at an unusually high frequency, and can extend from two mismatches (57–59). With such an error-prone polymerase, tandem mutations may likely arise during a single event that is targeted to a strand break within a hot-spot sequence. At the protein level, there seems to be no obvious advantage or disadvantage to having tandem DNA mutations vs. single mutations in changing codons.

Conclusions

There were fewer mutated heavy chains expressed by peripheral blood B cells from old people compared to young people, indicating that the immune response declines with age. However, among the mutated V genes, the frequency and pattern of mutation were similar between the two groups, which suggests that people can mount an adequate humoral response to antigens well into the ninth decade of life. This was confirmed by strong selection for mutations that changed amino acids in the CDRs in clones from old humans. There was no change in the overall frequency of microsatellite alterations in lymphocyte DNA with age; although there was variability on an individual level. A correlation between the frequency of microsatellite alterations and tandem mutations in V genes suggests that individuals vary in their DNA mismatch repair capacity.

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